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Laser microbeam-induced fixation for electronmicroscopy: Visualization of transient developmental features in nematode embryos

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Summary. In order to study development of embryos of *Caenorhabditis elegans* at an ultrastructural level, a new method of fixation has been developed. With a laser microbeam coupled to a microscope the impermeable eggshell is punctured to allow penetration of the fixative. At specific stages of embryogenesis further development can be arrested at will under visual control. As fixation occurs instantaneously, transient events (e.g. different phases of mitosis and cytokinesis) can be visualized.
Key words. Electronmicroscopy; laser; embryogenesis; mitosis; germline.

Cellular development of the nematode *Caenorhabditis elegans* has been extensively studied under the light microscope. The complete cell lineages from fertilization to adulthood have been described including cell fates[1-4]. In addition, electronmicroscopical analysis of various stages of development and of various parts of the hatched organism has been performed [5-10].

Because the embryo of *C. elegans* develops so reproducibly we are able to predict reliably the fate of each cell at any stage of development[4]. Thus, it is desirable to arrest individual embryos at specific stages for ultrastructural analysis of selected cells.

During early embryogenesis many typical events occur which are transient (e.g. pronuclear migration[11], pseudocleavage[11], asymmetric growth of membranes[10], formation of cleavage spindles[12] and segregation of germline-specific granules[13,14]). Because of their short duration and the impermeability of the eggshell it is difficult to fix an embryo exactly at the desired stage.

To facilitate this, it is necessary to observe the developing embryo under the microscope and then, at the chosen time, induce an instantaneous fixation of the egg for preservation of the structures of interest.

Here we describe a new method for fixation of the embryo at given developmental stages using a laser microbeam coupled to a microscope.

The developing embryos are dissected out of the mother in a drop of filter-sterilized water. Early embryos, well before the stage at which they will be fixed, are selected under a dissecting microscope and transferred with a drawn-out pasteur pipette to

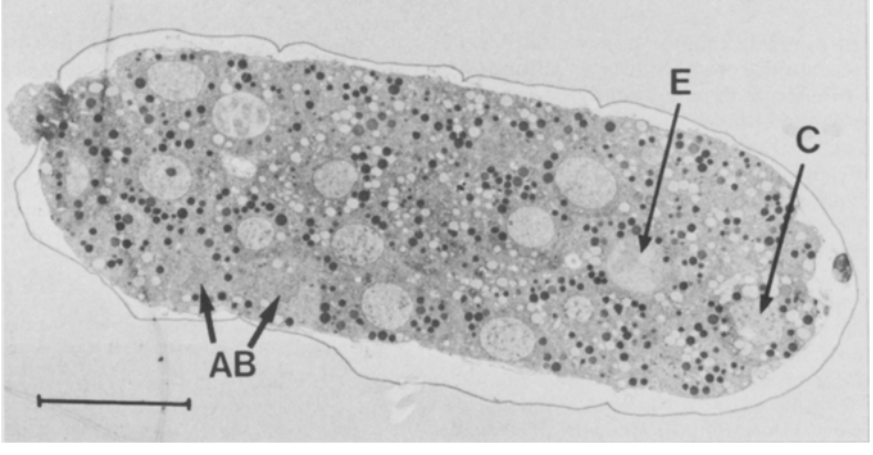
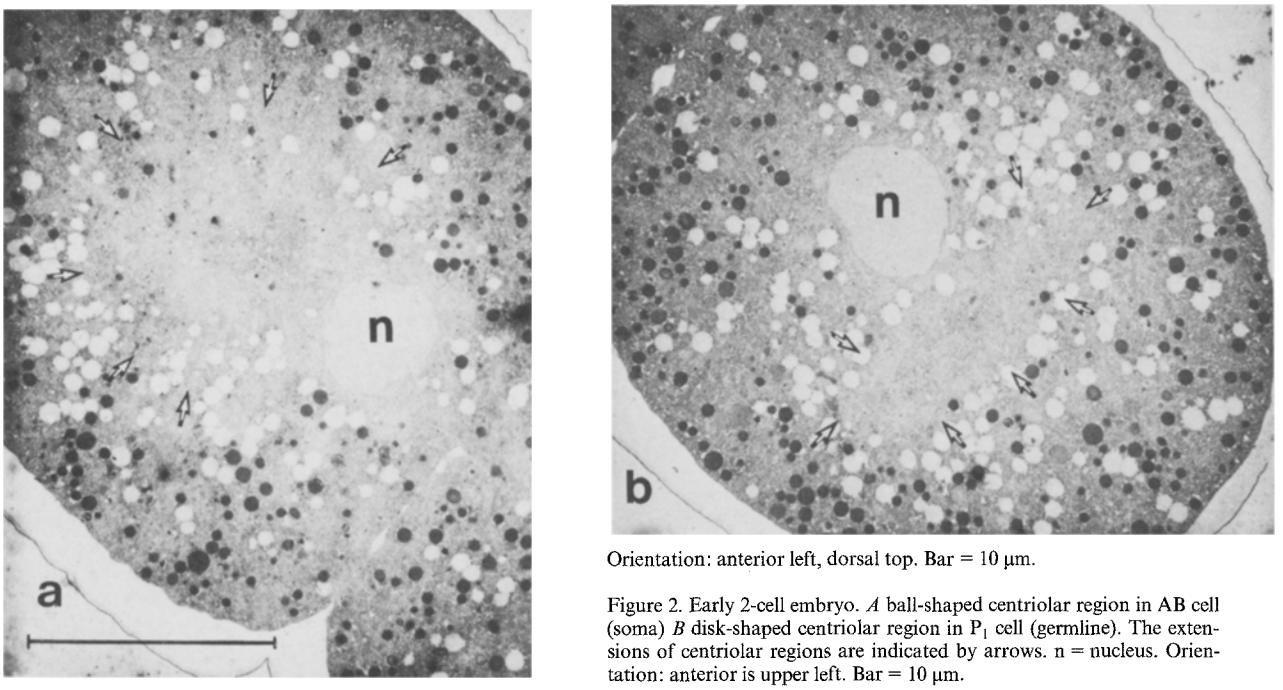


Figure 1. Longitudinal section through a 101-cell embryo after laser-induced fixation. At the point of laser penetration some cytoplasm has leaked out. Arrows point to nuclei which have been taken as landmarks to identify cells. AB = pharyngeal precursor in division, C = body muscle precursor in early mitosis, E = gut precursor at the onset of mitosis. Orientation: anterior left, dorsal top. Bar = 10 µm.



Orientation: anterior left, dorsal top. Bar = 10 µm.

Figure 2. Early 2-cell embryo. *A* ball-shaped centriolar region in AB cell (soma) *B* disk-shaped centriolar region in P_1 cell (germline). The extensions of centriolar regions are indicated by arrows. n = nucleus. Orientation: anterior is upper left. Bar = 10 µm.

a polylysine-coated microscope slide[15,16]. The coated slides are prepared in the following way: heat 100 ml of H_2O dest. to 60 °C, add 0.2 g gelatin, cool to 40 °C, add 0.02 g $CrK(SO_4)_2 \cdot 12\ H_2O$, add 100 mg of polylysine (MW 300,000). Clean microscope slide, add 1 drop of solution, spread, dry (e.g. with hair blower). The solution can be used for several weeks if stored in the refrigerator.

The transferred eggs will stick very reliably to the coated surface of the microscope slide. The water is replaced with medium containing 1-3% of glutaraldehyde. For our purpose the medium consists of 60% BM 86 Wissler (Boehringer, Mannheim), 15% Dulbecco's MEM (Serva), 15% Ham's F12 (Serva) and 10% fetal calf serum (Conco) at pH 7.2. Added to the medium is a drop of a saturated solution of Trypan blue in PBS, so that the medium shows a dark blue color. Eggs and medium are covered with a 24 × 24 mm coverslip and sealed with vaseline. As the fixative cannot pass the impermeable eggshell, development of the embryos continues undisturbed.

The slide is transferred to a compound microscope and development of eggs is observed with a 100 × objective under Nomarski optics. Coupled to the microscope is a nitrogen-pumped dye laser, which has been described earlier[17]. Trypan blue lightly stains the eggshell and allows the laser beam to be absorbed[18]. Those eggs which have reached the desired developmental stage are punctured with a single pulse of light using the orange laser dye Rhodamine 6G (Lambda Physics, Göttingen) with a wavelength of 645 nm. This allows the penetration of glutaraldehyde through the shell: cytoplasmic movement stops immediately and transient structures like mitotic spindles and growing cell membranes are preserved.

The microscope slide is transferred to the dissecting microscope and the coverslip is gently lifted. The medium is replaced with 3% glutaraldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 and the microscope slide is put in a humid chamber for about 1 h. The slide is then washed twice in sodium cacodylate buffer. Fixed eggs are easily identified because their development has been arrested while the others have continued to divide. The eggs of interest can be removed from the microscope slide with a drawn-out pasteur pipette. Eggs are individually transferred onto a new microscope slide coated with a layer of 4% agar (dissolved in sodium cacodylate buffer) and are covered with a small drop of warm agar. Then, blocks of agar containing single eggs are cut out and postfixed in 1% OsO_4 in the same buffer as above for 2 h at 4 °C, and further processed for electronmicroscopy[10,19].

Figure 1 shows a section through a 101-cell embryo, fixed with the method described above. After computer-aided reconstruction of serial sections all cells of this embryo have been identified[20]. This was relatively easy because a suitable developmental stage had been chosen for the time of fixation. Those few descendants of the somatic founder cell 'AB' which are in the process of cell division have been used as helpful landmarks. Thus, each cell could be labelled with its individual lineage name in order to compare light microscopical lineage data with the ultrastructural information.

Figure 2 shows sections of an early 2-cell embryo at the end of the first mitosis. The centriolar region of the somatic cell 'AB' shows a ball-like shape while the corresponding area in the germline cell 'P_1' has become flattened and forms a disk. This typical shape (which can be also observed in the following asymmetric division) persists in vivo for only a short time (a minute or so) before the structure is disassembled. Thus a precise timing of fixation is absolutely necessary to allow the ultrastructural analysis of this and other transient events.

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Allozyme polymorphism and linkage disequilibrium of *Adh* and *α-Gpdh* loci in wine cellar and field populations of *Drosophila melanogaster*

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Summary. Over three years, the *Adh* and *α-Gpdh* loci have been studied in two cellar populations of *Drosophila melanogaster* and in two field populations which were each near to one of the cellars. Analyses of gene frequencies indicate that the divergence among subpopulaitons is greater in the *Adh* locus than in the *α-Gpdh* locus. Selection for or against Adh^S allele acting on the *In(2L)t* inversion influences of the *α-Gpdh* alleles. This phenomenon may contribute to explain the maintenance of the *Adh* and *α-Gpdh* polymorphism and of the *In(2L)t* inversion.
Key words. Allozyme polymorphism; linkage disequilibrium; wine cellar and field populations; *Drosophila melanogaster*.

One selectionist explanation for the maintenance of the *Adh* locus polymorphism is that of frequency-dependent selection[1], although some authors feel that convincing evidence is lacking[2]. Laboratory experiments in which alcohol was added to the nutrient medium[3] indicate that the Adh^{FF} genotype is better adapted, whereas research in which individuals are submitted to temperature shocks[4] indicates that the Adh^{SS} genotype is better adapted to a higher temperature. The *α-Gpdh* locus codes for an essential enzyme in the energy metabolism of the fly and has been studied to determine the adaptative consequences of the existence of null alleles[5], and whether the maintenance of its polymorphism can be explained by frequency-dependent selection[6]. Linkage disequilibrium has been found between the *Adh* and *α-Gpdh* loci in populations from Japan and Texas[7]; the authors conclude that it is improbable that drift is a cause of these disequilibria; they postulate similar selective environments (ecological and genetic) in the two populations as the cause. On the other hand, the inversion-allozyme linkage is considered as a historical accident maintained by a heterotic effect in the fluctuating environment[8], while the polymorphism of two genes is maintained by random drift in natural populations. Yamaguchi et al.[9] have found a) that the linkage disequilibrium between these two loci is not induced by epistasis but by genetic drift, b) that linkage disequilibria between isozyme genes and polymorphic inversion are destined to disappear after many generations, and c) that linkage disequilibria due to immigration cannot be neglected in continuous populations.

Two wine cellar and two field populations from the southern Iberian Peninsula were analyzed. Samples were taken from each population at the end of the summer over a period of 3 years. 1200 flies were analyzed electrophoretically for *Adh* and *α-Gpdh* loci[10]. The wine cellar populations live in uniform environments where food is abundant in the form of yeast (impregnated by 12–15% alcohol), and where the annual average temperature is between 15 and 20 °C with daily oscillations of ± 1 °C. The field populations live in a variable environment where the annual variation of average temperature is between 10 and 25 °C with daily oscillations of ± 10 °C. For chromosomal analysis, 60 males were crossed with homozygous females of standard arrangement, and 7 larvae of the F_1 progeny were analyzed.

Table 1 shows a substantial deficiency of heterozygotes at the *Adh* locus in the two field populations, whilst the two wine cellar populations are close to equilibrium with a higher frequency of the Adh^F allele. The *α-Gpdh* locus shows a smaller (but still significant) deficiency of heterozygotes in field populations where the $\alpha\text{-}Gpdh^F$ allele is more abundant, and shows equilibrium H-W in wine cellars with the same frequency of the two alleles. The mean frequency of the Adh^F allele for the 1200 individuals is 0.6596 and that of the $\alpha\text{-}Gpdh^F$ allele is 0.6362. The two frequency values are significantly near to equality although they differ significantly for 0.5. Analysis of variation between populations within years (table 2a) shows values significantly different from zero for F, Ø and $f^{[1]}$ for the two loci in the 3 years, except the F values for the *α-Gpdh* locus in the 2nd year. The correlation Ø, which is a measure of the divergence between subpopulations, indicates a greater difference in the *Adh* locus between subpopulations (e.g. field vs wine cellar), with a high homozygosity in the subpopulations which is the cause of the high correlation between genes within individuals (F,f). The *α-Gpdh* locus shows the same annual variation as the *Adh* locus but with significantly lower values for F and f correlations. The Ø correlation (which is Wahlund's variance) can reach values significantly different from zero in two cases: 1) if populations are drifting because of small N_e or 2) if there is an interaction between the selection and the ecological habitat. In the first case, it must be taken into account that migration would reduce the Ø values to 0.2, if there is 1 migrant per subpopulation and per generation ($Ø = 1/1+4Nm$), and at the same time migration will reduce F; so, F would reach value near to zero. In the 2nd case, F and Ø values would be elevated and so would have intermediate values. As can be seen in table 2a, it seems that F, Ø and f values for *α-Gpdh* locus would be consistent with the first assumption, and those for *Adh* locus could be close to the second one. It must be taken into account that population structure influences high values of f, thus, the mixture of subpopulations increases the f value.

Analysis of variation between genes within subpopulations (table 2b) shows that F and f are greater in field than in wine cellar (as Comstock's test indicates) and also F and f are greater for *Adh* than for the *α-Gpdh* locus, (as can be seen from the results of Fisher's *z*-method). The Ø value is homogeneous for the four populations and it reaches the same value for the *Adh* locus as for the *α-Gpdh* locus; this is a reasonable result taking into account that the analysis has been performed for the 3 years within a same population. This homogeneity indicates that the changes produced in the subpopulations between years are not significant for both loci and for both environments (wine cellar, field). The high values of F in field are due to the existence for a